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# Evolution of Fungicide Resistance in the Cereal Eyespot Fungi *Tapesia yallundae* and *Tapesia acuformis* in France*

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**Abstract:** Field isolates of the cereal eyespot pathogen can be divided into two groups which are now considered as two species: *Tapesia yallundae* and *Tapesia acuformis*. In both species the first case of acquired resistance was observed with benzimidazole fungicides in the early 1980s. At the same time, a number of sterol C-14 demethylation inhibitors (DMIs), such as the imidazole prochloraz and several triazoles, including flusilazole, were introduced. Surprisingly *T. acuformis* appeared intrinsically resistant to the triazole derivatives in comparison to *T. yallundae*, but both species were sensitive to prochloraz. The intensive use of these DMIs led to the development of acquired resistance towards triazoles in *T. yallundae* and towards prochloraz in *T. acuformis*. Today all the strains in both species appear equally sensitive to the anilinopyrimidine cyprodinil.

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## 1 INTRODUCTION

Cereal eyespot is a damaging disease of autumn-sown wheat and barley crops in several European countries including France.[1] This disease is caused by two pathogens which are now considered as separate species: *Tapesia yallundae* and *Tapesia acuformis* (teleomorph stages).[2,3] They were previously classified as two varieties or types of the same species *Pseudocercosporella herpotrichoides* (Fron) Deighton (anamorph). Consequently, *T. yallundae* corresponds to the variety *herpotrichoides* and the SF-, L-, I- or W-types, whereas *T. acuformis* corresponds to the variety *acuformis* and the FE-, N-, II- or R-types.[3–5]

Conidia produced on infected debris represent the primary inoculum and can contaminate the cereals from autumn to spring. They are dispersed over short distances in rain-splash droplets. Eyespot is often considered as a mono-cycle disease; secondary infections originating from conidia produced on plant lesions can occur, but seem unimportant.[1] The role of ascospores, produced by apothecia, in the epidemiology remains to be determined.

After infection of the coleoptile or outer leaf sheath by conidia, the pathogen colonizes the plant tissues and it can penetrate successive sheaths. When stem extension has begun, the fungus can spread from the innermost leaf sheath to the stem. According to the extent of stem colonization, slight to severe lesions are recorded.

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Yield losses result from a reduced movement of water and nutrients through the damaged stem bases and from lodging.[1]

The most common way to control eyespot consists of a fungicide spray, between the end of tillering (GS 29) and the second node stage (GS 32). The active ingredient must inhibit the fungus inside plant tissues and/or prevent it spreading from the innermost leaf sheath to the stem. Treatment is recommended when more than 20% of the plants bear eyespot lesions at GS 29 or when positive responses are given by immunodiagnostic assays.[6,7] Today three groups of fungicides have been developed for the control of cereal eyespot. During more than ten years the most active and economical fungicides have been the antimicrotubular benzimidazoles (especially carbendazim). In the early 1980s, because of the selection of resistant strains, they were replaced by sterol C-14 demethylation inhibitors (DMIs) such as prochloraz (imidazole) or flusilazole (triazole). Recently, these DMIs were also affected by resistance and the anilinopyrimidine cyprodinil was introduced.[8]

The aim of this paper is to describe the in-vitro responses of the various fungal strains towards these three groups of fungicides and to present the practical consequences of the variability in eyespot fungi.

## 2 EXPERIMENTAL METHODS

All the tested strains were collected in France on winter wheat. They were maintained on malt agar or corn meal agar and conidia were produced in continuously illuminated cultures.[9]

The in-vitro effects of fungicides (technical grades) towards mycelial growth and germ-tube elongation were tested according to the methods described previously.[9,10] For each fungitoxicant, the concentration causing a 50% reduction of the mycelial growth ($EC_{50}$ mycelium) or of the length of germ-tubes ($EC_{50}$ germ-tubes) was determined using dosage-response curves.

For field trials, conducted on autumn-sown wheat crops, the experimental procedure was that recommended by the French C.E.B.[11] They included four replicates per treatment and each elementary plot measured at least 30 $m^2$. The fungicides were generally sprayed at first node stage (GS 31). The eyespot severity was evaluated after the flowering stage (GS 71–75) by assessing on 50–100 stems per treatment, the cross-sectional area colonized by the eyespot fungi. Additionally, strains of *T. yallundae* and/or *T. acuformis* were isolated from eyespot lesions (30 diseased stems per condition). Their phenotypes were determined as described previously.[10,12] Each field trial was identified by two numbers and a letter: the first number gave the date of the harvest, the second one represented the French Department and the letter corresponded to the location.

## 3 RESULTS AND DISCUSSION

### 3.1 Acquired resistance to benzimidazole fungicides in eyespot fungi

In both *T. yallundae* and *T. acuformis* three main types of benzimidazole-resistant strains were distinguished: (Table 1)

- *Ben1*$^+$ strains highly resistant to the benzimidazoles carbendazim and thiabendazole but highly sensitive to the phenylcarbamate diethofencarb;
- *Ben2*$^+$ strains highly resistant to carbendazim, moderately resistant to thiabendazole and sensitive to diethofencarb;
- *Ben3*$^+$ strains resistant to carbendazim, thiabendazole and diethofencarb.

From the survey conducted in France between 1984 and 1988 (within the benzimidazole-resistant strains), the respective percentages of *Ben1*$^+$, *Ben2*$^+$ and *Ben3*$^+$ phenotypes were 65, 25 and 10 in *T. yallundae* and 64, 23 and 13 in *T. acuformis*.[4] In both species, the most common benzimidazole-resistant strains, *Ben1*$^+$ and *Ben2*$^+$, exhibited similar base-pair mutations in the gene encoding for beta-tubulin (the target-site of benz-

**TABLE 1**
In-vitro Effects of Antimicrotubular Fungicides in the Eyespot Fungi *Tapesia yallundae* and *Tapesia acuformis*

| | *Extreme $EC_{50}$ mycelium values (mg litre$^{-1}$) for isolate* | | | |
|---|---|---|---|---|
| *Fungicides* | *Ben*$^-$ | *Ben1*$^+$ | *Ben2*$^+$ | *Ben3*$^+$ |
| Carbendazim | $<0{\cdot}1$ | $>25$ | 15–25 | $\geqq 5$ |
| Thiabendazole | 0·15–0·30 | $\geqq 25$ | 1·5–2·0 | $\geqq 5$ |
| Diethofencarb | $>10$ | 0·25–0·35 | $<0{\cdot}1$ | $>10$ |

**TABLE 2**
Structures of Populations of Eyespot Fungi Collected in the Control Plots of Eight Field Trials

| *Trials* | *Stems with mixtures*[a] (%) | T. acuformis[c] (%) | Ben$^{+}$ [b] *within* | |
|---|---|---|---|---|
| | | | T. yallundae (%) | T. acuformis (%) |
| 85-60A | 50 | 53 | 100 | 100 |
| 85-72A | 30 | 24 | 38 | 88 |
| 85-86A | 39 | 28 | 0 | 0 |
| 89-35A | 27 | 46 | 93 | 100 |
| 89-35B | 56 | 48 | 5 | 100 |
| 92-51A | 40 | 65 | 100 | 100 |
| 92-51B | 23 | 51 | 62 | 100 |
| 95-35C | 29 | 42 | 34 | 100 |

[a] *T. yallundae* and *T. acuformis* isolated from the same stem lesion.
[b] *Ben*$^{+}$, benzimidazole-resistant strains (*Ben1*$^{+}$, *Ben2*$^{+}$ and *Ben3*$^{+}$; see Section 3.1).
[c] % *T. acuformis* in the plot sample.

**TABLE 3**
In-vitro Effects of Sterol Biosynthesis Inhibitors towards Wild-Type Strains of *Tapesia yallundae* and *Tapesia acuformis*

| *Fungicides* | *Extreme* $EC_{50}$ *values* (*mg litre*$^{-1}$) | | | |
|---|---|---|---|---|
| | *Germ-tube elongation* | | *Mycelial growth* | |
| | T. yallundae | T. acuformis | T. yallundae | T. acuformis |
| Prochloraz | 0·002–0·005 | 0·003–0·007 | 0·03–0·07 | 0·02–0·05 |
| Triflumizole | 0·05–0·08 | 6–12 | 0·50–1·5 | >25 |
| Bromuconazole | 0·02–0·03 | 0·60–1·0 | 0·10–0·30 | 1·5–2·5 |
| Cyproconazole | 0·03–0·07 | 6–10 | 0·20–0·40 | 10–15 |
| Epoxyconazole | 0·015–0·030 | 0·20–0·40 | 0·10–0·20 | 0·20–0·30 |
| Flusilazole | 0·005–0·010 | 0·15–0·30 | 0·03–0·08 | 0·30–0·50 |
| Hexaconazole | 0·004–0·008 | 0·50–0·80 | 0·04–0·10 | 1·5–2·5 |
| Triadimenol | 0·20–0·50 | ≧10 | 0·5–2·0 | >25 |
| Fenpropimorph | 0·20–0·40 | 0·03–0·06 | 7–15 | 0·7–2·0 |

**TABLE 4**
Field Efficacies of Prochloraz and Flusilazole towards Eyespot in Winter Wheat

| *Number of trials* | *Control plots* | | *Efficacy* (%) | |
|---|---|---|---|---|
| | T. acuformis (%) | *Pro*$^{+}$ [a] (%) | *Prochloraz*[b] | *Flusilazole*[b] |
| | 1986–1988 | | | |
| 10 | 9 | 0 | 59 | 61 |
| 14 | 72 | 0 | 73 | 32 |
| | 1992–1993 | | | |
| 9 | 41 | 3 | 63 | 48 |
| 8 | 91 | 63 | 10 | 21 |

[a] Pro$^{+}$: prochloraz-resistant strains (*Pro1*$^{+}$ and *Pro2*$^{+}$). Symbols represent phenotypes and not identified resistance alleles.
[b] The fungicides applied at the first node stage (GS 31) were prochloraz at 600 g ha$^{-1}$ (before 1990) or 450 g ha$^{-1}$ (after 1990) or flusilazole at 300 g ha$^{-1}$ (before 1990) or 250 g ha$^{-1}$ (after 1990).

**TABLE 5**
Effects of DMI Fungicides on Field Populations of Eyespot Fungi in Trials conducted before 1990

| | | *Percentages of the various phenotypes* | | |
|---|---|---|---|---|
| | | T. yallundae | | T. acuformis |
| *Trials* | *Treatments*[a] | $Tri^-$ | $Tri^+$ | $Pro^-$ |
| 85-51C | Control | 30 | 11 | 59 |
| | Flusilazole | 0 | 2 | 93 |
| | Prochloraz | 52 | 5 | 43 |
| 87-36A | Control | 100 | 0 | 0 |
| | Cyproconazole | 61 | 28 | 11 |
| 87-62A | Control | 47 | 0 | 53 |
| | Cyproconazole | 13 | 0 | 87 |
| 89-86B | Control | 76 | 12 | 12 |
| | Bromuconazole | 10 | 59 | 31 |
| | Prochloraz | 77 | 23 | 0 |

[a] The fungicides applied at the first node stage (GS 31) were bromuconazole (300 g $ha^{-1}$), cyproconazole (120 g $ha^{-1}$), flusilazole (300 g $ha^{-1}$) or prochloraz (600 g $ha^{-1}$).

imidazoles and phenylcarbamates). The codon 198 encoding glutamate in *Ben*$^-$ was changed to an alanine in *Ben1*$^+$ and to a glycine in *Ben2*$^+$ (C. Albertini, pers. comm.).

Since 1985, in most field trials followed by us, the majority of isolates were resistant to benzimidazoles (e.g., trials 85-60A, 89-35A, 92-51A; Table 2) but in a few cases resistance was not detected (e.g. trial 85-86A). Additionally, in some locations, where *T. yallundae* and *T. acuformis* were detected frequently on the same stem lesions, benzimidazole resistance was generalized in *T. acuformis*, whereas in *T. yallundae* benzimidazole-sensitive strains predominated (e.g. trials 85-72A, 89-35B, 95-35C). This last result suggests that, under similar fungicide programmes, the selection pressure exerted by benzimidazole fungicides was higher in *T. acuformis* than in *T. yallundae*.

### 3.2 Resistance to sterol C-14 demethylation inhibitors in *Tapesia acuformis*

The in-vitro tests conducted with wild-type isolates of eyespot fungi indicated that those belonging to *T. acuformis* were intrinsically less sensitive towards most DMIs that those from *T. yallundae*. This natural resistance was observed with the triazole derivatives and some imidazoles such as triflumizole, but not with prochloraz. Additionally, fenpropimorph, another type of sterol biosynthesis inhibitor, also allowed differentiation between the species, but it is more toxic to *T. acuformis* than to *T. yallundae* (Table 3). In field trials conducted before 1990, the efficacies of prochloraz and flusilazole were compared in locations where either *T. yallundae* or *T. acuformis* dominated. As shown in Table 4, both DMIs were equally active when *T. yallundae* was preva-

**TABLE 6**
In-vitro Effects of DMIs towards Various Strains of *Tapesia acuformis*

| | *Extreme $EC_{50}$ 'germ-tubes' values (mg litre$^{-1}$)* | | |
|---|---|---|---|
| *Fungicides* | $Pro^-$ | $Pro1^+$ | $Pro2^+$ |
| Prochloraz | 0·003–0·007 | 0·2–0·4 | 0·2–0·4 |
| Triflumizole | 6·0–12 | 0·5–1·5 | 5·0–8·0 |
| Bromuconazole | 0·6–1·0 | 0·4–0·8 | 0·7–1·0 |
| Cyproconazole | 6·0–10 | 1·5–3·0 | 3·0–4·0 |
| Epoxyconazole | 0·2–0·4 | 0·4–0·8 | 0·5–1·0 |
| Flusilazole | 0·15–0·30 | 0·4–0·7 | 0·7–1·3 |
| Hexaconazole | 0·5–0·9 | 0·2–0·4 | 1·0–1·2 |

**TABLE 7**
Effects of DMI Fungicides on Field populations of Eyespot Fungi in Trials conducted after 1990

| | | *Phenotypes* (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | T. yallundae | | | T. acuformis | | |
| *Trials* | *Treatments*[a] | Tri$^-$ | Tri1$^+$ | Tri2$^+$ | Pro$^-$ | Pro1$^+$ | Pro2$^+$ |
| 93-80A | Control | 18 | 21 | 0 | 34 | 27 | 0 |
| | Flusilazole | 0 | 3 | 0 | 30 | 37 | 30 |
| | Prochloraz | 3 | 6 | 0 | 6 | 55 | 30 |
| 94-51D | Control | 14 | 29 | 11 | 20 | 23 | 3 |
| | Epoxyconazole | 0 | 35 | 12 | 8 | 45 | 0 |
| | Prochloraz | 0 | 9 | 37 | 0 | 43 | 11 |
| 94-80B | Control | 3 | 23 | 0 | 27 | 43 | 4 |
| | Hexaconazole | 0 | 28 | 0 | 36 | 30 | 6 |
| | Prochloraz | 0 | 0 | 0 | 0 | 67 | 33 |

[a] The fungicides applied at the first node stage (GS 31) were epoxyconazole (187·5 g ha$^{-1}$), flusilazole (250 g ha$^{-1}$), hexaconazole (250 g ha$^{-1}$) or prochloraz (450 g ha$^{-1}$).

lent, whereas flusilazole was less effective than prochloraz when *T. acuformis* predominated.

The differences in activity between prochloraz and triazole derivatives towards *T. acuformis* were also seen when studying the evolution of its frequency after fungicide treatments. From four representative field trials conducted between 1985 and 1989, it appeared that the selection pressure exerted by cyproconazole or flusilazole (triazole derivatives) towards *T. acuformis* was higher than for prochloraz (Table 5).

Since 1991, prochloraz-resistant strains of *T. acuformis* have been observed in several French regions, especially in North, Picardie and Champagne.[12,13] In tests conducted on conidia, the resistance levels towards prochloraz of such *Pro*$^+$ strains were about 60. According to their responses towards triflumizole, two phenotypes were recognized: the most common one, *Pro1*$^+$, exhibited hypersensitivity to this imidazole, whereas *Pro2*$^+$ was inhibited to the same extent as the wild-type *Pro*$^-$ (Table 6). Concerning the triazole derivatives, according to their in-vitro effects towards *Pro*$^-$ and *Pro1*$^+$ strains, they appeared equally toxic to both phenotypes (e.g. bromuconazole), more toxic to *Pro1*$^+$ strains (e.g. cyproconazole, hexaconazole) or slightly less toxic to *Pro1*$^+$ strains (e.g. epoxyconazole, flusilazole) (Table 6). These differences between triazoles could explain the fact that in field trials, flusilazole and epoxiconazole, like prochloraz, selected *Pro1*$^+$ strains, whereas cyproconazole or hexaconazole did not (Table 7). Furthermore, in field trials conducted after 1992, the efficacies of both prochloraz and flusilazole were very low in locations with high frequencies of *Pro*$^+$ strains,

**TABLE 8**
In-vitro Effects of DMIs towards Various Strains of *Tapesia yallundae*

| | *Extreme $EC_{50}$ germ-tubes values (mg litre$^{-1}$)* | | |
|---|---|---|---|
| *Fungicides* | Tri$^-$ | Tri1$^+$ [a] | Tri2$^+$ |
| Prochloraz | 0·002–0·007 | 0·003–0·006 | 0·3–0·6 |
| Triflumizole | $<$0·10 | $\geqq$ 10 | 2·0–6·0 |
| Bromuconazole | $<$0·05 | 0·8–1·5 | 1·0–3·0 |
| Cyproconazole | $<$0·10 | 6·0–$>$10 | 4·0–7·0 |
| Epoxyconazole | $<$0·05 | 0·5–1·0 | 1·0–2·0 |
| Flusilazole | $<$0·01 | 0·1–0·2 | 0·8–1·0 |
| Hexaconazole | $<$0·01 | 0·8–1·2 | 1·0–2·5 |
| Triadimenol | $<$0·50 | $>$10 | $>$10 |

[a] The *Tri1*$^+$ strains were chosen from among the most resistant ones towards triadimenol ($EC_{50}$ mycelium $>$25 mg litre$^{-1}$ or $EC_{50}$ germ-tubes $>$10 mg litre$^{-1}$).

whereas the disease control was better when *Pro*$^-$ strains predominated (Table 4).

### 3.3 Resistance to sterol C-14 demethylation inhibitors in *Tapesia yallundae*

From the survey conducted since 1985, we were able to characterize strains of *T. yallundae* sensitive to prochloraz but more or less resistant to most other DMIs, especially triazole derivatives.[4,10] The in-vitro responses of some of these *Tri1*$^+$ strains (selected from among the most resistant ones) towards several DMIs are given in Table 8. Under field conditions, triazole fungicides were able to select these *Tri1*$^+$ strains (Table 5). Furthermore, in trials conducted in the Champagne and Centre regions, where *Tri1*$^+$ strains were often found,[13] the triazole fungicides did not perform well.[6] However, there was no relationship between fungicide efficacy and the percentages of *Tri1*$^+$ strains in the control plots (data not shown).

More recently (since 1992), a phenotype highly resistant to prochloraz and triazole derivatives (*Tri2*$^+$) has been detected in France (Table 8). These *Tri2*$^+$ strains had probably not yet led to decreases in DMI efficacy because their frequency was generally below 10%. However in a trial conducted in Champagne in 1994, prochloraz selected such *Tri2* strains (Table 7).

### 3.4 In-vitro variability in response to cyprodinil

Cyprodinil belongs to the anilinopyrimidine group, which also includes mepanipyrim and pyrimethanil. These fungicides interfere with aminoacid biosynthesis and inhibit the secretion of enzymes such as pectinases and cellulases, involved in the pathogenic process.[14] When tested in water agar, cyprodinil inhibited the germ-tube elongation of all the tested strains of *T. yallundae* and *T. acuformis* at low concentrations ($EC_{50}$ values between 0.003 and 0.006 mg litre$^{-1}$). On the other hand, a variability in response to cyprodinil was observed in tests conducted on mycelium; the highest differences (even within a phenotype) occurred with a medium containing yeast extract (Table 9). Such an irregular and reduced toxicity of anilinopyrimidines when incorporated in complex media was previously recorded in *Botrytis cinerea* Pers. ex Fr.[15] Consequently, according to our observations and those of Heye *et al.*,[8] it seems that the only reliable in-vitro methods for testing sensitivity of field populations of eyespot fungi require the use of a 'poor' medium inoculated with conidia.

## 4 CONCLUSION

Eyespot of wheat, caused by two fungal species, *T. yallundae* and *T. acuformis*, is a monocycle disease, dispersed over short distances, which is treated only once a year. Nevertheless fungicide resistance appears as a major problem in many places in France.[13]

The acquired resistance to benzimidazoles, detected in the early 1980s and now generalized in both *T. yallundae* and *T. acuformis*, led to the official withdrawal of these fungicides (against eyespot) in 1991.

Towards the DMIs containing a triazole ring (e.g. bromuconazole, epoxyconazole, flusilazole) over several years, the natural resistance within *T. acuformis* and the (quantitative?) acquired resistance within *T. yallundae* accounted for irregular efficacies of these fungicides. In the case of the imidazole prochloraz the practical resistance, detected five or six years ago in North-West France,[13,16] was related to the development of a (qualitative) acquired resistance in *T. acuformis*. Recently, several mixtures combining prochloraz with a triazole derivative have been introduced. Their better efficacies were observed in regions where *T. yallundae* prevailed,[16] their intensive use could select strains resistant to both prochloraz and triazoles (*Tri2*$^+$ type).

Consequently, today, the most reliable solution consists in spraying cyprodinil, which seems to be equally active towards all the strains in both *T. yallundae* and

**TABLE 9**
In-vitro Effects of Cyprodinil on Eyespot Fungi

| | *Extreme $EC_{50}$ values (mg litre$^{-1}$)* | | | |
|---|---|---|---|---|
| | T. yallundae | | T. acuformis | |
| *Tests*[a] | Tri$^-$ | Tri$^+$ | Pro$^-$ | Pro$^+$ [b] |
| Germ-tube elongation | 0·003–0·006 | 0·003–0·006 | 0·003–0·006 | 0·003–0·006 |
| Mycelial growth [+ YE] | 0·15–>10 | 0·2–3·0 | 0·15–>10 | 0·15–10 |
| Mycelial growth [− YE] | 0·2–0·8 | 0·07–1·0 | 0·03–0·08 | 0·03–0·08 |

[a] In the mycelium tests, the fungi were cultivated on media in the absence [− YE] or presence [+ YE] of yeast extract.
[b] In *T. yallundae*, *Tri*$^+$ included *Tri1*$^+$ and *Tri2*$^+$ while in *T. acuformis Pro*$^+$ included *Pro1*$^+$ and *Pro2*$^+$.

*T. acuformis*. However we must be watchful, because its intensive use could lead to the selection of resistant strains as observed in *B. cinerea.*[17]

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